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Zebularine Induces Long-Term Survival of Pancreatic Islet Allotransplants in Streptozotocin Treated Diabetic Rats

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Abstract

Background: Coping with the immune rejection of allotransplants or autologous cells in patients with an active sensitization towards their autoantigens and autoimmunity presently necessitates life-long immune suppressive therapy acting on the immune system as a whole, which makes the patients vulnerable to infections and increases their risk of developing cancer. New technologies to induce antigen selective long-lasting immunosuppression or immune tolerance are therefore much needed.

Methodology/Principal Findings: The DNA demethylating agent Zebularine, previously demonstrated to induce expression of the genes for the immunosuppressive enzymes indolamine-2,3-deoxygenase-1 (IDO1) and kynureninase of the kynurenine pathway, is tested for capacity to suppress rejection of allotransplants. Allogeneic pancreatic islets from Lewis rats were transplanted under the kidney capsule of Fischer rats previously made diabetic by a streptozotocin injection (40 mg/kg). One group was treated with Zebularine (225 mg/kg) daily for 14 days from day 6 or 8 after transplantation, and a control group received no further treatment. Survival of the transplants was monitored by blood sugar measurements. Rats, normoglycemic for 90 days after allografting, were subjected to transplant removal by nephrectomy to confirm whether normoglycemia was indeed due to a surviving insulin producing transplant, or alternatively was a result of recovery of pancreatic insulin production in some toxin-treated rats. Of 9 Zebularine treated rats, 4 were still normoglycemic after 90 days and became hyperglycemic after nephrectomy. The mean length of normoglycemia in the Zebularine group was 67 ± 8 days as compared to 14 ± 3 days in 9 controls. Seven rats (2 controls and 5 Zebularine treated) were normoglycemic at 90 days due to pancreatic recovery as demonstrated by failure of nephrectomy to induce hyperglycemia.

Conclusions/Significance: Zebularine treatment in vivo induces a long-lasting suppression of the immune destruction of allogeneic pancreatic islets resulting in protection of allograft function for more than 10 weeks after end of treatment.

Introduction

There is an increasing clinical need for replacing malfunctioning organs or cells producing key substances by transplantation from allogeneic donors or from the patient himself. Although donor matching can minimize the histocompatibility barrier in the former case [1], life-long immunosuppressive medication is at present required to protect against immune rejection of the transplant [2]. Even in the case of transplantation of syngeneic cells, e.g., after cells have been genetically engineered to produce insulin in a patient with type-1 diabetes, a similar long-term immunosuppressive treatment will be needed to protect the transplanted cells from destruction by immune responses to the auto-antigens that originally caused the disease [3]. Presently, coping with the immune reactivity in these and similar cases will necessitate life-long immune suppressive therapy acting on the immune system as a whole, which is known to make the patients vulnerable to infections and to increase their risk of developing cancer [4].

A dramatic improvement will come when it will be possible to induce a long-lasting, antigen selective immunosuppression or tolerance to the key antigens in question for each individual patient. Besides avoiding life-long therapy, the most important advantage will be that there will be no induction of general immune suppression with its accompanying sensitivity to infections and enhanced cancer risk.

Zebularine is a methyl transferase inhibitor that in dividing cells will be incorporated into the DNA as a deoxy base replacing the deoxyctydine. Since it is a cytidine analog, it will function as deoxyctydine in the DNA when being transcribed by the RNA polymerases [5]. When Zebularine is incorporated into the DNA, it will inhibit the DNA methylases [5]. However, all methylated
cytidine analogs will not be demethylated [6–7]. In studies of the
effect of Zebularine on the antigenicity of tumor cells [8], we
found that treatment with a moderately high dose of Zebularine
(100 μM) increased the expression of the gene of the enzyme
indolamine-2,3-dioxygenase-1 (IDO1) in rat colon cancer cells
in vitro and drastically reduced their immunogenicity. This effect
on immunogenicity could be inhibited by the competitive inhibitor
1-methyl-tryptophan, which indicates that the Zebularine-induced
inhibition of the tumor immunogenicity was indeed caused mainly
by IDO1. In addition, Zebularine has been shown to induce a
strong expression of IDO1 in rat bone marrow-derived dendritic
cells generated ex vivo (unpublished data). Although the process of
demethylation is to some extent probably random, our subsequent
microarray studies on normal, polyclonally stimulated human
peripheral monocytes revealed that several of the genes in the
tryptophan pathway were preferentially upregulated in the
presence of Zebularine, especially IDO1 and kynureninase (own
unpublished data). It is conceivable that in each cell type there
might exist a hierarchy of genes that are more prone than others to
be demethylated in this manner. Subsequently, we have demon-
strated a strong synergism between Zebularine and interferon
gamma (IFN-γ) in inducing prolonged IDO1 expression in human
monocytic THP-1 cells [7], (unpublished data).

On the basis of these results we propose to use Zebularine as a
novel conditioning treatment of recipients of transplants and a
therapy for patients with autoimmune diseases. IDO1 and
kynureninase are key enzymes in the catabolism of the essential
amino acid tryptophan. It is well established that IDO1 is one of
the key molecules expressed by tolerogenic dendritic cells both in
experimental animals and in humans [9]. The basis for the
suppressive effect on T-cells has been shown to be the extreme
sensitivity of T-cells to tryptophan starvation [10] and the
apoptotic effect of the catabolites [11]. Recently, data were
presented that strongly suggested that the IDO1 molecule,
independent of its enzymatic effect, also can function as a signal
transducer for transforming growth factor beta (TGF-β1), another
key immunosuppressive molecule expressed by many cell types
including tolerogenic dendritic cells [12].

Taking into account the vital role of IDO1 for immunological
tolerance, and the findings that Zebularine treatment induces
IDO1 expression, such treatment might be a powerful tool for
preventing immunogenic reactions against transplanted tissues and
controlling autoimmune reactions by turning these into a
tolerogenic state. In the present study we take a first step to
establish this novel mode of immunosuppression by demonstrating
that Zebularine treatment in vivo is capable of suppressing a
strong immune rejection response towards pancreatic islet
alloqrafts differing from the recipient with regard to portions of
major histocompatibility antigens [13].

Materials and Methods

Ethics Statement

All animal procedures were performed according to the practices of the Swedish Board of Animal Research and were
approved by the Swedish Ethics Committee, Lund-Malmö,
Sweden (Permit number: M310-10). All efforts were made to
minimize suffering.

Animals

Lewis rats, Fischer 344 rats and Wistar rats were obtained from
Charles River Laboratories (Germany) and kept at a 12-h light
and 12-h dark cycle in rat hutchies (2–3 in each cage) with free
access to standard rodent chow and tap water.

All surgery was performed under isoflurane inhalation anaes-
thesia. No antibiotics were used. Buprenorphine (Temgesic,
Schering-Plough, NJ, USA) was administered intramuscularly
(0.05 mg/kg) after islet transplantation and nephrectomy to
alleviate postoperative pain. After finishing the experiments, the
rats were sacrificed by opening of the thoracic cavity and emptying
of the inferior vena cava from blood under isoflurane anesthesia.

Zebularine Treatment

The cytidine analogue Zebularine was obtained from Ber-
ry&Associates (Dexter, MI, USA) and dissolved in PBS (50 mg/
ml). Zebularine (225 mg/kg) was administered by intraperitoneal
injection. The dose is atoxic and does not affect the general
condition of the rats. The dose has been shown to induce an
increased IDO1 expression in rat spleens (unpublished data).

Before starting the allotransplantation study a pilot study was
performed to verify that the Zebularine dose chosen would induce
increased IDO1 expression in vivo. In this study male Wistar rats
received daily treatment with Zebularine (225 mg/kg, 2 rats) or
PBS (controls, 2 rats) for 7 days. The rats were sacrificed the day
after the last Zebularine injection (day 8) and the spleens isolated
and frozen in liquid nitrogen for isolation of RNA.

RNA Isolation

Pieces of frozen spleens were homogenized in liquid nitrogen
using mortar and placed in TRIZol reagent (Invitrogen, Life
Technologies, Paisley, UK), and then total RNA was extracted
corresponding to Invitrogen’s protocol. Quality and quantity of
isolated RNA was measured by gel electrophoresis and specto-
photometer.

Quantitative Real Time PCR Analysis

The qRT-PCR analyses were performed using the SuperScript
III Platinum Two-Step qRT-PCR Kit with SYBR Green
(Invitrogen, Life Technologies, Paisley, UK). A total of 1 μg total
RNA was used in a 20 μl RT reaction using a mixture of polydT
and random hexamers primers. The cDNA obtained was diluted
to a total volume of 80 μl and stored at −20°C.

The primer sequences for the different genes were designed
using Gene Fisher software support (G. Giegerich, F. Meyer, C.
Schleier-macher, ISMB-96). The primers used for amplification
of the rat IDO1 (rIDO1) gene were 5'-GGTTCTTCTGATA-
TTTGTCCGG-3' (forward) and 5'-CAGGGGGGAGTT-
CAGGCGCA-3' (reverse) according to a rat cDNA sequence
from a PCR product 248 bp in size. For amplification of
the endogenous reference gene hypoxanthine guanine phosphoribosyl
transferase (HPRT) forward primer 5'-CAAGCTTGGCTGT-
GAAAAAGG-3' and a reverse primer 5'-CACAAAGAT-
GATTCA-AATCCCCTGA-3', according to the cDNA sequence,
were used. The qRT-PCR was performed in 20 μl reaction
consisting of 2 μl diluted cDNA, 0.3 μM of each primer,
1×bovine serum albumin (50 μg/ml) and 10 μl Platinum SYBR
Green qRT-PCR superMix-UDG. The amplification of rIDO1
and HPRT were carried out in a Light Cycler (Roche Molecular
Biochemical). The amplification of rIDO1 was carried out with
the following thermal profile: UDG incubation at 35°C for 2 min,
then denaturation at 95°C for 5 min, followed by 45 cycles at
94°C for 6 s, 56°C for 10 s, and 72°C for 14 s. The amplification
of rHPRT was carried out as follows: UDG incubation at 35°C for
2 min, then denaturation at 95°C for 5 min, followed by 45 cycles
at 94°C for 6 s, 57 for 10 s, and 72°C for 14 s. After amplification
a melting curve analysis was performed. The qRT-PCR exper-
iments were run in triplicate.
Data analysis was performed with Light Cycler software version 3 (Roche Molecular Biochemicals). The threshold level was determined using the software, according to optimization of the baseline and the standard curve. Standards were obtained by amplification of a control DNA sample in a RT-PCR, using the same primers, reagents, and conditions optimized for the real-time analysis. The IDO1 expression levels were normalized with the expression levels of HPRT.

**Induction of Diabetes**
Streptozotocin (STZ) was obtained from Sigma-Aldrich Sweden AB (Stockholm, Sweden) and dissolved in PBS. A single dose of STZ (Sigma-Aldrich Sweden AB, Stockholm, Sweden) was administered intraperitoneally to recipient rats to induce diabetes. Blood glucose was monitored using a Bayer Contour glucometer (Bayer AB, Diabetes Care, Solna, Stockholm). Before performing the transplantation study, the dose of STZ needed to establish a stable diabetic state was determined. At moderately high doses, 30–45 mg/kg i.p., the rats (16 rats in total) developed diabetes, with blood glucose levels ≥20 mmol/L. None of the animals with induced diabetes returned to euglycemia. Our results are in line with previous studies [14–15], where a STZ dose of 30–40 mg/kg induced diabetes returned to euglycemia. Our results are in line with previous studies [14–15], where a STZ dose of 30–40 mg/kg induced diabetes returned to euglycemia. Our results are in line with previous studies [14–15], where a STZ dose of 30–40 mg/kg induced diabetes returned to euglycemia.

**Islet Transplantation**
Islets from one to two donors were used.

**Histopathology**
After dissection, the relevant kidneys were immersion-fixed in 4% buffered formalin and stored in 70% ethyl-alcohol for up to two months before being embedded in paraffin. The kidneys were serially sectioned at 5 micrometer in a plane perpendicular to the long axis of the organ. Every tenth section of the kidneys was stained with Haematoxylin and Eosin and examined in the microscope; sections containing an obvious transplant or a distinctive lesion were further analyzed and adjacent sections were immunohistochemically processed for the visualization of cells containing insulin.

These sections were deparaffinized and treated in a microwave oven for retrieval of antigen before being incubated with a mouse monoclonal antibody against insulin (AM20632PU-N) (Acris Antibodies GmbH, Herford, Germany) at a dilution of 1:200 for two hours and subsequently with a biotinylated secondary antibody and ABC reagent 30 minutes each, these two later steps were carried out using a ready-to-use Vectastain ABC kit (Vector Laboratories, CA, USA) where after the antigen-antibody complex was visualized using the DAKO Liquid DAB Substrate-Chromogen System (DAKO, CA, USA) and the sections were stained with Haematoxylin and Eosin. The sections were thoroughly rinsed in PBS before each step of the immunohistochemical procedure.

The sections were analyzed by an observer blinded to the experimental protocol using a Zeiss light-microscope utilizing 12.5× eyepieces and a 20× EC plan-neofluar objective giving a field of view of 0.6 mm².
The number of immunopositive cells within the transplant was counted as the number of immunopositive cellular profiles with a visible nucleus observed within the one visual field exhibiting the largest number of cells (− = no cells; + = 1–10 cells; ++ = 11–20 cells; +++ ≥ 20 cells).

Statistics
2-sided Mann-Whitney test was applied, as well as Kaplan-Meier analysis, for analysis of survival. Pearson correlation analysis was used to compute the correlation coefficient.

Results
Systemic Treatment with Zebularine Induces IDO1 in Rat Spleens
Systemic daily treatment of male Wistar rats with Zebularine (225 mg/kg) for 7 days induced a two- to fivefold increase in IDO1 expression in spleens, as compared to rats treated with PBS (Figure 1).

Syngeneic Fischer-to-Fischer Transplantation
For syngeneic Fischer to Fischer transplantation, 500–900 islets cured the diabetic state induced by exposure to STZ. Removal of the islet transplant after more than 3 months of euglycemia by nephrectomy lead to hyperglycemia in 3 out of 4 cases. This was taken as proof that the induced normoglycemia in these 3 rats was dependent on the grafted islets. All 3 animals also had insulin-containing cells detectable under the kidney capsule. In the fourth case, graft removal did not lead to hyperglycemia, a recovery of pancreatic insulin production that is known to occur in a proportion of rats exposed to STZ [17–19]. In this animal insulin-containing cells were detected in the kidney. This illustrates the necessity to check whether a long-lasting normoglycemic state is dependent on transplant survival or recovery of pancreatic insulin production.

Prolonged Survival of Pancreatic Islet Allografts in Rats Treated with Zebularine
Rats normoglycemic for 90 days after allografting were subjected to transplant removal by nephrectomy to confirm whether euglycemia was indeed due to a surviving insulin producing transplant, or alternatively was a result of recovery of pancreatic insulin production (see above). A total of 7 rats (2 controls and 5 Zebularine treated) were normoglycemic at day 90 due to pancreatic recovery as demonstrated by failure of nephrectomy to induce hyperglycemia and these rats were excluded from further evaluation. Of the remaining 9 Zebularine treated rats, 4 were still euglycemic after 90 days and became hyperglycemic after nephrectomy (histological scoring +++ for three of them (see Figure 2) and ++ for one). The mean length of time the Zebularine group stayed normoglycemic was 67±8 (SEM) days after transplantation as compared to 14±3 (SEM) days for the 9 controls (Figure 3). This difference is highly significant (p<0.001, 2-sided Mann-Whitney). Median length of euglycemia was 84 days in the Zebularine treated group and 11 days in the control group. In the five Zebularine treated animals, which developed hyperglycemia before the end of the 90 days observation period was reached indicating rejection of the allograft, although delayed, there were remaining insulin expressing cells in 2 animals (histological scoring of ++ for both of them, diabetic at days 15 and 37) (Figure 4) but in 3 there were no such cells detectable (diabetic at days 51, 56 and 84).

Nine control animals developed hyperglycemia during the observation period. Eight of these were analyzed. Six of the animals had no insulin positive staining in their kidneys (Figure 5), whereas two rats had remaining insulin expressing cells in the kidney scoring as ++ or +++ (diabetic first at day 43 after transplantation, Figure 6).

There was no significant correlation between the amount of transplanted islets, and survival (Pearson coefficient −0.28 for the controls and −0.35 for the Zebularine treated animals, p-value n.s.).

Discussion
In this study, we show that the DNA demethylating agent Zebularine has a strong suppressive effect on the rejection response against Lewis rat pancreatic islets allotransplanted under the kidney capsule of diabetic Fischer rats. The survival of the transplants was monitored by frequent blood sugar measurements and a confirmed value >10.0 was regarded as the time point when

Figure 1. Expression of IDO1 in rat spleen.

Analysis of the IDO1-gene expression in spleens from four Wistar rats treated with daily i.p. injections of Zebularine (225 mg/kg/day, two rats) or PBS (two rats) for 7 days. IDO1 expression was measured by Real-time qPCR fluorescence and the normalized rIDO1 expression level for each spleen sample calculated by rIDO1 expression divided by rHPRT expression. The normalized IDO1 expressions of spleens from Zebularine-treated rats are presented in relation to the mean of that of PBS-treated controls.
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the transplanted islets had been rejected and the insulin levels were no longer sufficient to maintain normoglycemia. Rats that were still normoglycemic at 90 days after allografting were nephrectomized to determine whether their normal blood sugar was a result of the survival of the transplant, or was instead due to the recovery of insulin production in the pancreas. Such a recovery is to be expected in a proportion of rats after exposure to Streptozotocin [17–19]. Animals being normoglycemic after nephrectomy were excluded from evaluation since the survival of their islet allograft could not be adequately determined. The mean survival time of the allograft as judged in this way was highly significantly prolonged in the Zebularine treated group compared to the controls receiving no therapy after the allotransplantation (mean of 67±8 days vs 14±3 days, p<0.001). Out of 9 Zebularine treated rats maintaining their grafts for 90 days without developing hyperglycemia, 4 were shown by nephrectomy to still require their allografted islets to maintain their normoglycemic state. The other 5 rats remained normoglycemic upon graft removal and were consequently excluded from evaluation because of recovery of the pancreatic insulin production.

Kidneys which had received transplants of allogeneic pancreatic islets were investigated immunohistologically for the presence of

![Figures 2. Survival of transplant in Zebularine treated rat at the end of the 90-days observation period. Left: Insulin positive cells (red-brown) of the transplant (T) are seen superficial to the normal renal cortex (RC) (semiquantitative scoring of ++). A large collection of leucocytes (L) is buried deep into the transplanted tissue. Bar is 400 μm. Right: The area outlined in a higher magnification; insulin positive cells are easy to distinguish from adjacent leucocytes and renal parenchyma. Bar is 80 μm. doi:10.1371/journal.pone.0071981.g002](image)

![Figure 3. Euglycemic survival after allotransplantation. Days of euglycemia for animals which finally developed hyperglycemia (nine Zebularine treated animals out of which four were hyperglycemic after nephrectomy at day 90 versus nine control animals) (p<0.001, 2-sided Mann Whitney). Zebularine treated animals as black line, and control animals as white-and-black line. doi:10.1371/journal.pone.0071981.g003](image)
cells expressing insulin. As expected, all the 4 Zebularine treated rats that remained normoglycemic for 90 days were found to have insulin expressing cells in the kidney. Among the 5 Zebularine treated rats that became hyperglycemic within 90 days, 2 also had remaining insulin expressing cells in their kidneys, although obviously not sufficiently many to maintain normoglycemia. No insulin containing cells could be detected in the other 3 rats. Eight of the 9 controls were also investigated and while 6 had no detectable insulin expressing cells, 2 had remaining insulin containing cells, although obviously not sufficient in quantity to maintain a normoglycemic state.

Based on our previous analyses, the mechanism of action of this novel mode of immunosuppressive treatment with Zebularine is the induction of expression of the rate limiting tryptophan catabolizing enzyme IDO1. This occurs preferentially in certain subsets of dendritic cells, initiating the kynurenine pathway. The dose of Zebularine used in the present study was chosen on the basis of our previous finding that daily treatment with Zebularine (225 mg/kg, i.p.) for three weeks induces a significantly enhanced expression of IDO1 in the spleen as demonstrated by PCR (unpublished data). In the present study, this was verified in healthy control rats before starting the allotransplantation study. Here, daily treatment with Zebularine (225 mg/kg, i.p.) for 7 days
induced a two- to fivefold increase in IDO1 expression in the spleen (Figure 1).

In at least some cells, including dendritic cells, Zebularine activates expression of IDO1 and kynureninase. These enzymes have been reported to suppress T lymphocytes by creating local tryptophan shortage [10] and excess concentrations of tryptophan catabolites [11]. Among cytokines IFN-γ is the strongest inducer, although in vitro the effect disappears rapidly after removal of the cytokine to return to the base level within approximately 24 h (unpublished data). We have demonstrated a very strong synergistic induction of IDO1 expression by Zebularine and IFN-γ in the human monocytic cell line THP-1 in vitro (unpublished data). This synergistic effect is also long-lasting and is observed more than a week after removal of the IFN-γ. This is of particular interest since IFN-γ is commonly expressed in areas of graft rejection and inflammatory sites in patients with autoimmunity, in which Zebularine might consequently be expected to induce a synergistic, particularly strong IDO1 expression. In addition to its enzymatic action to catabolize tryptophan, it was recently reported that the IDO1 molecule can also function as a signal transducer for TGF-β1 [12], enabling this cytokine to activate the alternative NFκB pathway. Through this pathway TGF-β1 may induce its own production and the expression of IDO1 as well. This might have the potential of creating a long-lasting immunosuppressive loop through the generation of regulatory T lymphocytes via the effect of TGF-β1 and T-cell inhibition via the IDO1.

An analogously suppressed allorejection has previously been reported after IDO1 gene transfer into the tissue to be grafted [20] and this has been interpreted to be due to a T-cell inhibitory effect of the IDO1. Furthermore, a similar transfer of the IDO1 gene into bone marrow derived dendritic cells and subsequent use of these cells to treat mice with collagen induced rheumatic arthritis (RA) has been reported to strongly suppress the inflammatory disease process in this murine RA model [21–22]. At the present time, however, an increased risk of cancer development by this type of gene transfer and gene manipulation cannot be ruled out, why they are mainly reserved for life-threatening diseases.

The results of our present investigation are important, representing the first demonstration that systemic administration of the IDO1- and kynureninase-inducing drug Zebularine is causing a long-lasting suppression of allograft rejection as a sign of immunosuppression and possibly immune tolerance. The results indicate that Zebularine might provide a tool by which a short period of drug therapy enables the permanent survival of allografted cells and organs, such as pancreatic islets. A similar protection against autoimmune destruction might be anticipated for autologously grafted, insulin-producing cells differentiated from stem cells, when the technology to produce them has been sufficiently developed. The mode of using Zebularine, alone or in combination with other modulators of immune responsiveness, is to be further explored both technically and in relation to application on autoimmunity. This will include expansion of the work on using dendritic or other cells after in vitro exposure of these cells to Zebularine, relevant antigens, and/or other immune cells as a mean to enhance antigen selectivity, efficiency and safety.

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Author Contributions

Conceived and designed the experiments: HN PE KF ZX GS BW HOS LGS. Performed the experiments: HN PE KF SS LJ ZX GS BW HOS LGS. Analyzed the data: HN PE KF SS LJ ZX GS BW HOS LGS. Contributed reagents/materials/analysis tools: HN PE KF SS LJ ZX GS BW HOS LGS. Wrote the paper: HN PE KF SS LJ ZX GS BW HOS LGS.
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